invitrogen™
by life technologies™

pSecTag/FRT/V5-His TOPO® TA Expression Kit

For five-minute cloning of *Taq* polymerase-amplified PCR products into a vector for secreted expression in the Flp-In™ System

Catalog no. K6025-01

Version E
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A Limited Label License covers this product (see Purchaser Notification). By use of this product, you accept the terms and conditions of the Limited Label License.

User Manual

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Important Information

Shipping and Storage

The pSecTag/FRT/V5-His TOPO® TA Expression Kit is shipped on dry ice. Each kit contains a box with pSecTag/FRT/V5-His TOPO TA Cloning® reagents (Box 1) and a box with One Shot® TOP10 competent cells (Box 2).

Store Box 1 at -20°C. Store Box 2 at -80°C.

TOPO TA Cloning® Reagents

pSecTag/FRT/V5-His TOPO TA Cloning® reagents (Box 1) are listed below. Note that the user must supply *Taq* polymerase. **Store Box 1 at -20°C.**

Item	Concentration	Amount
pSecTag/FRT/V5-His-TOPO® vector, linearized	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	--	1 ml
T7 Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
BGH Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Expression Control Plasmid (pSecTag/FRT/V5-His/PSA)	0.5 µg/µl in TE buffer, pH 8	10 µl
Control PCR Primers	0.1 µg/µl each in TE Buffer, pH 8	10 µl
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8	10 µl

continued on next page

Important Information, continued

Primer Sequences

The sequence of each primer is provided below:

Primer	Sequence	pMoles Supplied
T7	5´-TAATACGACTCACTATAGGG-3´	328
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	358

One Shot® TOP10 Reagents

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit. Transformation efficiency is at least 1 x 10^9 cfu/µg DNA. Note that One Shot® TOP10 cells may be ordered separately (Catalog no. C4040-03).

Store Box 2 at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype

TOP10: Use this strain for general cloning of PCR products in pSecTag/FRT/V5-His-TOPO®.

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

One Shot® Electrocomp™

TOP10 cells are also available as electrocompetent cells in a One Shot® format (Catalog no. C4040-52). Transformation efficiency is 1 x 10^9 cfu/µg supercoiled DNA.

Accessory Products

Introduction

The products listed in this section are intended for use with the pSecTag/FRT/V5-His TOPO® TA Expression Kit. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

Products Available Separately

Some of the products included in the pSecTag/FRT/V5-His TOPO® TA Expression Kit as well as other reagents that may be used with the Flp-In™ System are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
T7 Promoter Primer	2 µg, lyophilized in TE	N560-02
Hygromycin	1 g	R220-05
Zeocin™	1 g	R250-01
	5 g	R250-05
pFRT/*lac*Zeo	20 µg, lyophilized in TE	V6015-20
pFRT/*lac*Zeo2	20 µg, lyophilized in TE	V6022-20
pOG44	20 µg, lyophilized in TE	V6005-20
One Shot® Kit (TOP10 **Chemically Competent** Cells)	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® Kit (TOP10 **Electrocompetent** Cells)	10 reactions	C4040-50
	20 reactions	C4040-52

Flp-In™ Expression Vectors

Additional Flp-In™ expression vectors are available from Invitrogen. For more information about the features of each vector, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30). Ordering information is provided below.

Product	Amount	Catalog no.
pcDNA5/FRT	20 µg, lyophilized in TE	V6010-20
pcDNA5/FRT/V5-His TOPO® TA Expression Kit	1 kit	K6020-01
pEF5/FRT/V5 Directional TOPO® Expression Kit	1 kit	K6035-01
pEF5/FRT/V5-DEST Gateway™ Vector Pack	6 µg	V6020-20

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Accessory Products, continued

Flp-In™ Host Cell Lines

For your convenience, Invitrogen has available several mammalian Flp-In™ host cell lines that stably express the *lacZ-Zeocin*™ fusion gene from pFRT/*lac*Zeo or pFRT/*lac*Zeo2. Each cell line contains a single integrated FRT site as confirmed by Southern blot analysis. The cell lines should be maintained in medium containing Zeocin™. For more information, see our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

Cell Line	Amount	Catalog no.
Flp-In™-293	3 x 10^6 cells, frozen	R750-07
Flp-In™-CV-1	3 x 10^6 cells, frozen	R752-07
Flp-In™-CHO	3 x 10^6 cells, frozen	R758-07
Flp-In™-BHK	3 x 10^6 cells, frozen	R760-07
Flp-In™-3T3	3 x 10^6 cells, frozen	R761-07
Flp-In™-Jurkat	3 x 10^6 cells, frozen	R762-07

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The table below describes the antibodies available for detection of C-terminal fusion proteins expressed using pSecTag/FRT/V5-His-TOPO®. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods.

Fifty microliters of each antibody is supplied which is sufficient for 25 Westerns.

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991) GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25

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Accessory Products, continued

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using Invitrogen's ProBond™ Resin (see below). To purify proteins expressed from pSecTag/FRT/V5-His-TOPO®, the ProBond™ Purification System or the ProBond™ resin in bulk are available separately. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Metal-Binding Resin	50 ml	R801-01
	150 ml	R801-15
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 kit	K854-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The pSecTag/FRT/V5-His TOPO® TA Expression Kit combines the Flp-In™ System with TOPO® Cloning technology to provide a highly efficient, rapid cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for targeted and secreted expression of the gene of interest in mammalian cell lines. TOPO Cloning® requires no ligase, post-PCR procedures, or PCR primers containing special, additional sequences. For more information about TOPO® Cloning, see the next page.

pSecTag/FRT/V5-His-TOPO® Vector

pSecTag/FRT/V5-His-TOPO® is a 5.2 kb expression vector designed to facilitate rapid cloning and expression of PCR products using the Flp-In™ System (Catalog nos. K6010-01 and K6010-02) available from Invitrogen. When cotransfected with the pOG44 Flp recombinase expression plasmid into a Flp-In™ mammalian host cell line, the pSecTag/FRT/V5-His-TOPO® vector containing the PCR product of interest is integrated in a Flp recombinase-dependent manner into the genome. The pSecTag/FRT/V5-His-TOPO® vector contains the following elements:

- The human cytomegalovirus (CMV) immediate-early enhancer/promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
- Murine Ig κ-chain leader sequence for directing secreted expression of the gene of interest (Coloma *et al.*, 1992)
- TOPO® Cloning site for rapid and efficient cloning of *Taq*-amplified PCR products (see the next page for more information)
- C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag for detection and purification of recombinant protein
- FLP Recombination Target (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-In™ host cell line (see pages 2-3 for more information)
- Hygromycin resistance gene for selection of stable cell lines (Gritz and Davies, 1983) (see important note on page 3)

The control plasmid, pSecTag/FRT/V5-His/PSA, is included for use as a positive control for transfection and secreted expression in the Flp-In™ host cell line of choice.

For more information about the Flp-In™ System, the pOG44 plasmid, and generation of the Flp-In™ host cell line, refer to the Flp-In™ System manual. The Flp-In™ System manual is supplied with the Flp-In™ Complete or Core Systems, but is also available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 30).

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Overview, continued

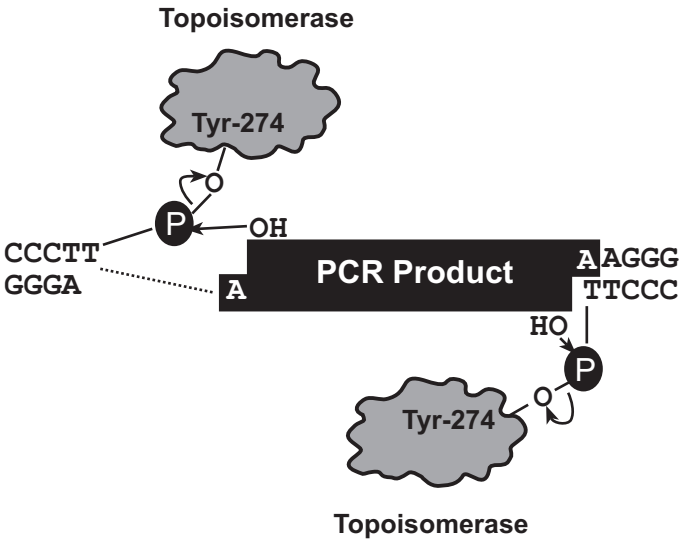
How TOPO® Cloning Works

The plasmid vector, pSecTag/FRT/V5-His-TOPO®, is supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase covalently bound to the vector (this is referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products (see below).



FRT Site in the pSecTag/FRT/V5-His-TOPO® Vector

The pSecTag/FRT/V5-His-TOPO® vector contains a single FRT site immediately upstream of the hygromycin resistance gene for Flp recombinase-mediated integration and selection of the pSecTag/FRT/V5-His-TOPO® construct following cotransfection of the vector (with pOG44) into a Flp-In™ mammalian host cell line. The FRT site serves as both the recognition and cleavage site for the Flp recombinase and allows recombination to occur immediately adjacent to the hygromycin resistance gene. The flp recombinase is expressed from the pOG44 plasmid. For more information about the FRT site and recombination, see the next page. For more information about pOG44, refer to the pOG44 manual or the Flp-In™ System manual.

continued on next page

Overview, continued

Flp Recombinase-Mediated DNA Recombination

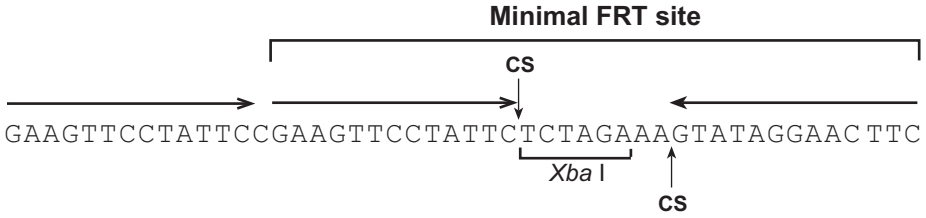
In the Flp-In™ System, integration of your pSecTag/FRT/V5-His-TOPO® expression construct into the genome occurs via Flp recombinase-mediated intermolecular DNA recombination. The hallmarks of Flp-mediated recombination are listed below.

- Recombination occurs between specific FRT sites (see below) on the interacting DNA molecules
- Recombination is conservative and requires no DNA synthesis; the FRT sites are preserved following recombination and there is minimal opportunity for introduction of mutations at the recombination site
- Strand exchange requires only the small 34 bp minimal FRT site (see below)

For more information about the Flp recombinase and conservative site-specific recombination, refer to published reviews (Craig, 1988; Sauer, 1994).

FRT Site

The FRT site, originally isolated from *Saccharomyces cerevisiae*, serves as a binding site for Flp recombinase and has been well-characterized (Gronostajski and Sadowski, 1985; Jayaram, 1985; Sauer, 1994; Senecoff *et al.*, 1985). The minimal FRT site consists of a 34 bp sequence containing two 13 bp imperfect inverted repeats separated by an 8 bp spacer that includes an *Xba* I restriction site (see figure below). An additional 13 bp repeat is found in most FRT sites, but is not required for cleavage (Andrews *et al.*, 1985). While Flp recombinase binds to all three of the 13 bp repeats, strand cleavage actually occurs at the boundaries of the 8 bp spacer region (see figure below) (Andrews *et al.*, 1985; Senecoff *et al.*, 1985).



CS = cleavage site

Important

The hygromycin resistance gene in pSecTag/FRT/V5-His-TOPO® lacks a promoter and an ATG initiation codon; therefore, transfection of the pSecTag/FRT/V5-His-TOPO® plasmid alone into mammalian host cells will **not** confer hygromycin resistance to the cells. The SV40 promoter and ATG initiation codon required for expression of the hygromycin resistance gene are integrated into the genome (in the Flp-In™ host cell line) and are only brought into the correct proximity and frame with the hygromycin resistance gene through Flp recombinase-mediated integration of pSecTag/FRT/V5-His-TOPO® at the FRT site. For more information about the generation of the Flp-In™ host cell line and details of the Flp-In™ System, refer to the Flp-In™ System manual.

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Overview, continued

Experimental Outline

The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the N-terminal Ig κ-chain secretion signal and the C-terminal peptide containing the V5 epitope and the polyhistidine (6xHis) tag (if desired). Consult the diagram on page 6 to help you design your PCR primers.	5-6
2	Produce your PCR product.	7
3	TOPO® Clone your PCR product into pSecTag/FRT/V5-His-TOPO® and transform into One Shot® TOP10 *E. coli*. Select transformants on LB plates containing 50-100 μg/ml ampicillin.	8-10
4	Analyze your transformants for the presence and orientation of insert by restriction enzyme digestion.	11
5	Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the Ig κ-chain secretion signal and the C-terminal peptide.	11
6	Cotransfect your pSecTag/FRT/V5-His-TOPO® construct and pOG44 into the Flp-In™ host cell line using your method of choice and select for hygromycin resistant clones (see the Flp-In™ System manual for more information).	14-15
7	Assay for expression of your protein of interest.	16-17
8	Purify your recombinant protein by chromatography on metal-chelating resin (*e.g.* ProBond™).	18

Methods

PCR Primer Design

Introduction

It is important to properly design your PCR primers to ensure that you obtain the recombinant protein you need for your studies. Use the information below and the diagram on page 6 to design your PCR primers. **Remember that your PCR product will have single 3´ adenine overhangs.**

General Molecular Biology Techniques

For help with *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pSecTag/FRT/V5-His-TOPO®.

Fusion to the Ig κ-Chain Secretion Signal

In order to obtain proper secreted expression of your protein, you must design your 5′ PCR primer such that the PCR product will clone in frame with the initiation ATG of the N-terminal Ig κ-chain secretion signal.

Fusion to the C-terminal Peptide

If you wish to include the C-terminal peptide for detection with either the V5 or His (C-term) antibodies or purification using the polyhistidine (6xHis) tag, you must design your reverse PCR primer to remove the native stop codon and maintain the frame through the DNA encoding the C-terminal peptide.

If you do not wish to include the C-terminal peptide, include the native stop codon in the reverse PCR primer or design the primer to anneal downstream of the native stop codon.

Note: Cloning efficiencies may vary depending on the 5′ nucleotide sequence of your primer (see page 26).

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to page 7.

Signal Sequence Processing

The Ig κ-chain secretion signal is processed from your recombinant protein by a signal peptidase-directed cleavage after aspartic acid 21 in the signal sequence. For the location of the signal cleavage site, refer to the diagram on the next page. Note that you will not obtain native protein following cleavage of the signal sequence because of the intervening sequences between the signal cleavage site and the beginning of your PCR product (see the next page).

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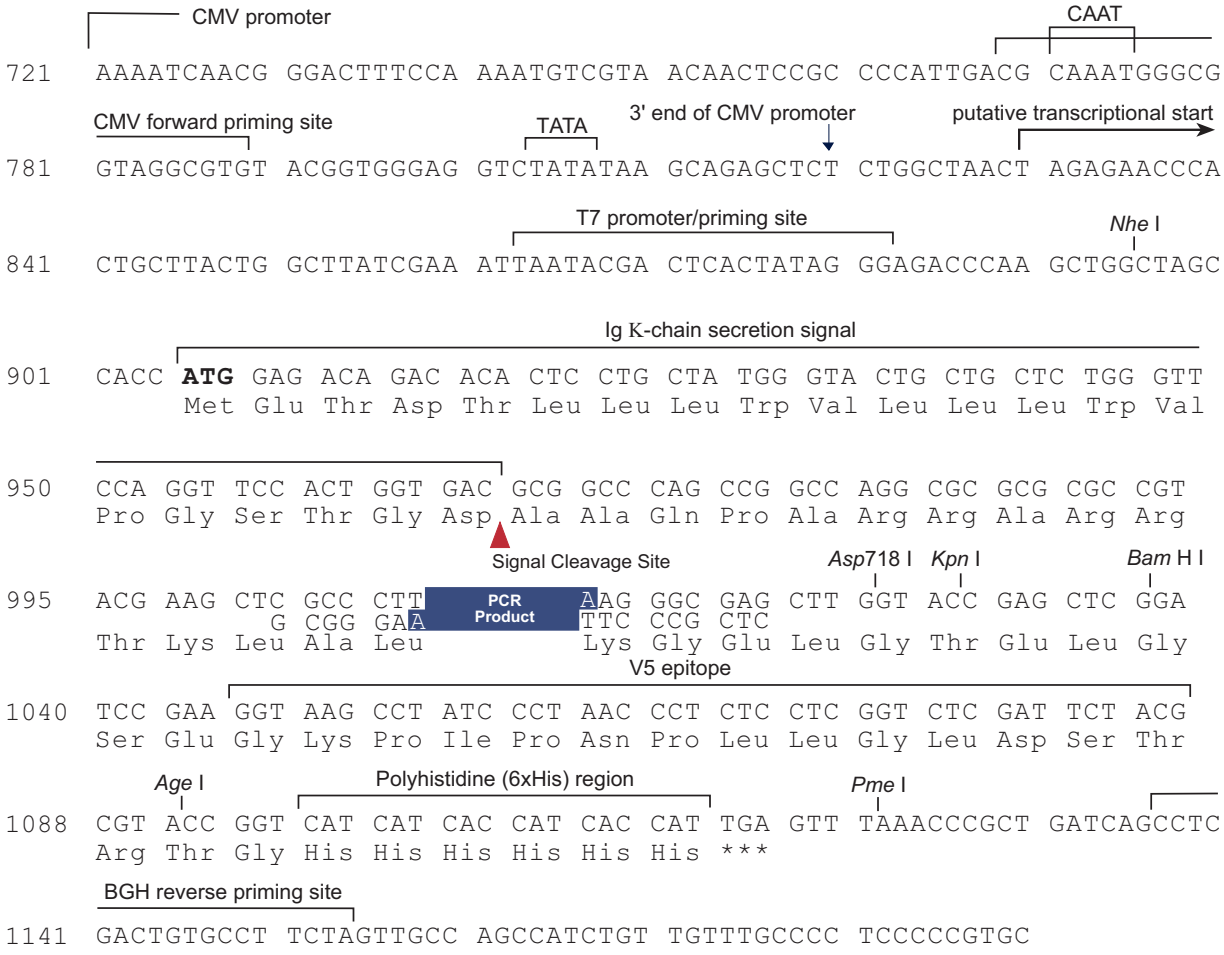
PCR Primer Design, continued

TOPO® Cloning Site for pSecTag/FRT/V5-His-TOPO®

The diagram below is supplied to help you design appropriate PCR primers to correctly clone and express your PCR product using pSecTag/FRT/V5-His-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 1012 and 1013. This is the TOPO® Cloning site. **The complete sequence of pSecTag/FRT/V5-His-TOPO® is available for downloading from our World Wide Web site (**www.invitrogen.com**) or from Technical Service (page 30).** For a map and a description of the features of pSecTag/FRT/V5-His-TOPO®, see the **Appendix**, pages 27-28.


CMV promoter
CAAT
721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG
CMV forward priming site
TATA
3' end of CMV promoter
putative transcriptional start
781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
T7 promoter/priming site
Nhe I
841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
Ig K-chain secretion signal
901 CACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG CTC TGG GTT
Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val
950 CCA GGT TCC ACT GGT GAC GCG GCC CAG CCG GCC AGG CGC GCG CGC CGT
Pro Gly Ser Thr Gly Asp Ala Ala Gln Pro Ala Arg Arg Ala Arg Arg
Signal Cleavage Site
Asp718 I
Kpn I
Bam H I
995 ACG AAG CTC GCC CTT AAG GGC GAG CTT GGT ACC GAG CTC GGA
PCR Product
G CGG GAA TTC CCG CTC
Thr Lys Leu Ala Leu Lys Gly Glu Leu Gly Thr Glu Leu Gly
V5 epitope
1040 TCC GAA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG
Ser Glu Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr
Age I
Polyhistidine (6xHis) region
Pme I
1088 CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTT TAAACCCGCT GATCAGCCTC
Arg Thr Gly His His His His His His ***
BGH reverse priming site
1141 GACTGTGCCT TCTAGTTGCC AGCCATCTGT TGTTTGCCCC TCCCCCGTGC

Producing PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product.

Materials Supplied by the User

You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proof-reading polymerase only, you can add 3′ A-overhangs using the method on page 23.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Primers (100-200 ng each)	1 µM each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the pSecTag/FRT/V5-His TOPO® TA Expression Kit (see page 21). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Call Technical Service for more information (page 30).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into pSecTag/FRT/V5-His-TOPO®, and transform the recombinant vector into TOP10 *E. coli* in one day. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 24-25 in parallel with your samples. If you have previously TOPO® Cloned, read the **Note** below.

Note

Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction results in the following:

- a 2- to 3-fold increase in the number of transformants.
- allows for longer incubation times (up to 30 minutes). Longer incubation times can result in an increase in the number of transformants obtained.

Including salt in the TOPO® Cloning reaction prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

If you do **not** include salt in the TOPO® Cloning reaction, the number of transformants obtained generally decreases as the incubation time increases beyond 5 minutes.

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see below).** For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results. Read the following information carefully.

Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing when electroporating. The Salt Solution provided in the kit must be diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see next page).

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TOPO® Cloning Reaction and Transformation, continued

Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50-100 μg/ml ampicillin (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis or sequencing for the presence and orientation of insert. Sequencing primers included in each kit can be used to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparation for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (*e.g.* add 5 μl of the Salt Solution to 15 μl sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 μl) for eventual transformation into either chemically competent One Shot® TOP10 *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 13.

Note: The red or yellow color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 μl	0.5 to 4 μl
Salt Solution	1 μl	--
Dilute Salt Solution	--	1 μl
Sterile Water	add to a final volume of 5 μl	add to a final volume of 5 μl
TOPO® vector	1 μl	1 μl

*Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or +4°C.

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

2. Place the reaction on ice and proceed to **One Shot® Chemical Transformation** (next page) or **Transformation by Electroporation** (next page). **Note**: You may store the TOPO® Cloning reaction at -20°C overnight.

One Shot® Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from Step 2 previous page into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion (see above).

3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 25-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

Transformation by Electroporation

1. Add 2 µl of the TOPO® Cloning reaction into a 0.1 cm cuvette containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see next page.

3. Immediately add 250 µl of room temperature SOC medium.
4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.
5. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Note

Addition of the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol-precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analysis of Positive Clones

1. Culture 10 transformants overnight in 2-5 ml LB or SOB medium containing 50-100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids for the presence and orientation of insert by restriction analysis. We recommend sequencing your constructs to confirm that your gene of interest is cloned in frame with the C-terminal peptide. Sequencing primers are included to help you sequence your insert (see page iv). Refer to the diagram on page 6 for the sequence surrounding the TOPO® Cloning site.

   If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. You may use either the forward **or** reverse sequencing primers included in the kit **and** a primer that hybridizes within your insert. You will have to determine the amplification conditions.

If this is the first time you have used this technique, we recommend that you perform restriction analysis in parallel to confirm that PCR gives you the correct result. Both false positive and false negative results can be obtained because of mispriming or contaminating template.

The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µl reaction volume. Multiply by the number of colonies to be analyzed (*e.g.* 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. (Don't forget to make a patch plate to preserve the colonies for further analysis.)
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using the appropriate conditions (see text above).
5. For the final extension, incubate at 72°C for 10 minutes. Hold at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

TOPO Cloning® Reaction and Transformation, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 24-25. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to isolate a single colony and prepare a glycerol stock for long term storage. We recommend that you also store the purified plasmid DNA at -20°C.

1. Streak the original colony on LB plates containing 50-100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product by precipitation

Transfection and Analysis

Introduction

Once you have TOPO® Cloned your gene of interest into pSecTag/FRT/V5-His-TOPO® and have prepared purified plasmid DNA of your expression construct and pOG44, you are ready to cotransfect the plasmids into your mammalian Flp-In™ host cell line to generate your stable Flp-In™ expression cell line. We recommend that you include the pSecTag/FRT/V5-His/PSA positive control vector (see the next page) and a mock transfection (negative control) in your experiments to evaluate your results. General information about transfection, selection, and expression analysis is provided in this section. Specific guidelines and protocols for generating the Flp-In™ expression cell line can be found in the Flp-In™ System manual.

For detailed information about pOG44 and generation of the Flp-In™ host cell line, refer to the Flp-In™ System manual. A separate manual for the pOG44 plasmid is also available from our Web site (www.invitrogen.com) or by calling Technical Service (see page 30).

Several Flp-In™ host cell lines which stably express the *lacZ-Zeocin*™ fusion gene and contain a single integrated FRT site are available from Invitrogen (see page vii for ordering information). If you wish to express your gene of interest in 293, CV-1, CHO, 3T3, BHK, or Jurkat cells, you may want to use one of the Flp-In™ host cell lines to establish your expression cell line. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

Important

We have observed down-regulation of the viral CMV promoter and subsequent loss of gene expression when pcDNA5/FRT-based expression constructs are introduced into 3T3 or BHK cells. This behavior is not observed with pEF5/FRT-based expression constructs. If you are generationg Flp-In™ expression cell lines using a 3T3 or BHK host cell line, we recommend that you clone your gene of interest into a pEF5/FRT-based expression plasmid (*e.g.* pEF5/FRT/V5-D-TOPO® or pEF5/FRT/V5-DEST). For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 30).

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa, CHO), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium require-ments, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Invitrogen offers the Calcium Phosphate Transfection Kit (Catalog no. K2780-01) and several lipid-based reagents for mammalian cell transfection. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

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Positive Control

pSecTag/FRT/V5-His/PSA is provided as a positive control vector for mammalian cell transfection and expression (see page 29 for a map) and may be used to assay for recombinant protein expression levels in your Flp-In™ host cell line. Cotransfection of the positive control vector and pOG44 into your Flp-In™ host cell line allows you to generate a stable cell line expressing prostate specific antigen (PSA) at the same genomic locus as your gene of interest. If you have several different Flp-In™ host cell lines, you may use the pSecTag/FRT/V5-His/PSA control vector to compare protein expression levels between the various cell lines.

To propagate and maintain the plasmid:

1. Resuspend the vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, JM109, or equivalent.
2. Select transformants on LB agar plates containing 50-100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Hygromycin B

The pSecTag/FRT/V5-His-TOPO® vector contains the hygromycin resistance gene (Gritz and Davies, 1983) for selection of transfectants with the antibiotic, hygromycin B (Palmer *et al.*, 1987). When added to cultured mammalian cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis. Hygromycin B liquid is supplied with the Flp-In™ Complete System and is also available separately from Invitrogen (Catalog no. R220-05). For instructions to handle and store hygromycin B, see the Flp-In™ System manual.

Determination of Hygromycin Sensitivity

Before generating a stable cell line expressing your protein of interest (Flp-In™ expression cell line), we recommend that you generate a kill curve to determine the minimum concentration of hygromycin required to kill your untransfected Flp-In™ host cell line. Generally, concentrations between 10 and 400 µg/ml hygromycin are required for selection of most mammalian cell lines. General guidelines for performing a kill curve are provided in the Flp-In™ System manual.

Important

REMINDER: Remember that the hygromycin resistance gene in pSecTag/FRT/V5-His-TOPO® lacks a promoter and an ATG initiation codon; therefore, transfection of the pSecTag/FRT/V5-His-TOPO® plasmid alone into mammalian host cells will **not** confer hygromycin resistance to the cells. The SV40 promoter and ATG initiation codon required for expression of the hygromycin resistance gene are integrated into the genome (in the Flp-In™ host cell line) and can only be brought into the correct proximity and frame with the hygromycin resistance gene through Flp recombinase-mediated integration of pSecTag/FRT/V5-His-TOPO® at the FRT site.

continued on next page

Transfection and Analysis, continued

Generation of Flp-In™ Expression Cell Lines

Refer to the Flp-In™ System manual for detailed guidelines and instructions to cotransfect your pSecTag/FRT/V5-His-TOPO® construct and pOG44 into the Flp-In™ host cell line to generate stable Flp-In™ expression cell lines. Once you have generated your Flp-In™ expression cell line, see the next page for general guidelines to assay for expression of your recombinant fusion protein.

Note

Your gene of interest will be expressed from pSecTag/FRT/V5-His-TOPO® under the control of the human CMV promoter. Once you have generated the Flp-In™ expression cell line, note that your recombinant fusion protein will be constitutively expressed.

Detection of Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use Anti-V5 antibodies or Anti-His(C-term) antibodies available from Invitrogen (see page vii for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

Detection of Secreted Protein from Medium

The medium in which your Flp-In™ expression cells are grown can be analyzed for secreted, recombinant fusion protein by functional assay or Western blot analysis. If you are also harvesting cells, see **Preparation of Cell Lysates**, next page. Before starting, prepare 2X SDS-PAGE sample buffer. A recipe is provided on page 20 for your convenience, but other recipes are suitable. If you are using pre-cast polyacrylamide gels (see the next page), refer to the manufacturer's instructions to prepare the appropriate sample buffer.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein.
2. Harvest the medium from the cells.

   Note: Depending on the sensitivity of your antibody, you may wish to concentrate the media samples prior to Western blot analysis. You may use any method to concentrate the media samples. We suggest using commercially available ultrafiltration devices (*e.g.* Centricon) or a Speed-Vac.
3. For each media sample, mix 20 µl of media with 20 µl of 2X SDS-PAGE sample buffer.
4. Boil the samples for 5 minutes. Centrifuge briefly.
5. Load samples, electrophorese, blot, and probe with a suitable antibody (see above).
6. Visualize proteins using your desired method.

Note

The amino acids between the Ig κ-chain secretion signal and the TOPO® Cloning site will add approximately 1.5 kDa to the size of your protein while the C-terminal tag containing the V5 epitope and the polyhistidine (6xHis) tag will add approximately 3.6 kDa to the size of your protein.

continued on next page

Preparation of Cell Lysates

Before starting, prepare Cell Lysis Buffer. A recipe is provided on page 19 for your convenience, but other recipes are suitable.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein
2. Remove the medium from each plate and prepare samples as detailed on the previous page.
3. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS, see the **Appendix**, page 20 for a recipe).
4. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
5. Resuspend in 50 µl Cell Lysis Buffer. Vortex.
6. Incubate cell suspension at 37°C for 10 minutes to lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
7. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. **Note:** Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
8. Add SDS-PAGE sample buffer (see page 20 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
9. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese (see the next page). Use the appropriate percentage of acrylamide to resolve your fusion protein.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 30).

Assay for PSA

If you use pSecTag/FRT/V5-His/PSA as a positive control vector, you may assay for PSA expression using your method of choice. We generally use the Total PSA Enzyme Immunoassay Test Kit (American Laboratory Products, Catalog no. 025-BC-1019) to assay for PSA expression.

Note that PSA is fused to the C-terminal peptide, so you can use Western blot analysis and either the Anti-V5 antibody or the Anti-His(C-term) antibody to detect expression of PSA. The PSA/V5-His protein fusion migrates around 31.5 kDa on an SDS-PAGE gel.

Purification

Introduction

Once you have generated your Flp-In™ expression cell line and have verified that your recombinant fusion protein expresses, you may use metal-chelating resin such as ProBond™ to purify the recombinant protein. General guidelines are provided below. For more details about purification using ProBond™, refer to the ProBond™ Purification System manual.

Purification of Secreted Recombinant Protein

To purify secreted, recombinant protein from the medium, follow the manufacturer's instructions for the metal-chelating resin that you are using. Start with about 3 to 5 ml of medium and load onto 1 to 2 ml of resin. Scale up or down depending on the level of expression and the capacity of your resin.

Purification of Recombinant Protein from Cells

You may also purify recombinant protein from cell lysates. In this case, you will need 5×10^6 to 1×10^7 transfected cells for purification of your protein on a 2 ml ProBond™ column (or other metal-chelating column). If you are using ProBond™ to purify your protein, refer to the protocol below to prepare cells for lysis. If you are using another metal-chelating resin, refer to the manufacturer's instructions to prepare your cells.

Preparation of Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™. You will need 5×10^6 to 1×10^7 stably transfected cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual for details).

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are approximately 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1500 x g for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 x g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at –70°C until needed.

Lysis of Cells

If you are using ProBond™ resin, refer to the ProBond™ Purification System manual for details about sample preparation for chromotography.

If you are using other metal-chelating resin, refer to the manufacturer's instructions for recommendations on sample preparation.

Note

If you are not using metal-chelating resin to purify your secreted, recombinant protein, we recommend that you culture the cells in serum-free medium or in reduced-serum medium to avoid or lessen the amount of bovine serum albumin present in your sample. Whether you are able to culture your cells in serum-free medium will depend on the nature of your cell line and the availability of commercial serum-free media formulations for the particular cell type. Refer to the supplier of your media or serum for more information.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add 50 μg/ml ampicillin if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add 50 μg/ml ampicillin and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.
5. To add X-gal to the plate, warm the plate to 37°C. Pipette 40 μl of the 40 mg/ml X-gal stock solution (see below), spread evenly, and let dry 15 minutes. Protect plates from light.

X-Gal Stock Solution

1. To prepare a 40 mg/ml stock solution, dissolve 400 mg X-Gal in 10 ml dimethyl-formamide.
2. Protect from light by storing in a brown bottle at -20°C.

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 μM leupeptin, or 0.1 μM aprotinin before use.

continued on next page

Recipes, continued

Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve: 8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   in 800 ml deionized water.
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter. You may wish to filter-sterilize or autoclave the solution to increase shelf life.

2X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2.0 ml
β-mercaptoethanol	0.4 ml
Bromophenol Blue	0.02 g
SDS	0.4 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.

Note

Note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 7).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE. Borate will interfere with the NaI step (Step 2.)
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M NaI.
3. Add 1.5 volumes of Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 9.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 9). Be sure to make the gel slice as small as possible for best results.

continued on next page

Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that the gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 9.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into chemically competent One Shot® TOP10 cells using the method on page 10.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO® Cloning vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the 3´ to 5´ exonuclease activity, which removes the 3´ A-overhangs necessary for TOPO® Cloning. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pSecTag/FRT/V5-His-TOPO®.

Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

Note

You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see page 21). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 µl in the TOPO® Cloning reaction.

Vent® is a registered trademark of New England Biolabs.

pSecTag/FRT/V5-His TOPO® Control Reactions

Introduction

If you have trouble obtaining transformants or vector containing insert, perform the following control reactions to help troubleshoot your experiment. Performing the control reactions involves producing a control PCR product containing the *lac* promoter and the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product in either direction will yield blue colonies on LB agar plates containing antibiotic and X-gal.

Before Starting

Be sure to prepare LB plates containing 50-100 µg/ml ampicillin **and X-gal** (see page 19 for recipe) before performing the control reaction:

Producing Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and LacZα, set up the following 50 µl PCR:

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

pSecTag/FRT/V5-His TOPO® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
TOPO® vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of TOP10 One Shot® cells (page 10).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin and X-Gal (see page 19). Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 85% of these will be blue.

The "vector only" plate should yield very few colonies (<15% of the vector + PCR insert plate) and these should be all white.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 10. Plate 10 µl of the transformation mixture plus 20 µl of SOC to help ensure even spreading on LB plates containing 50 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

continued on next page

pSecTag/FRT/V5-His TOPO® Control Reactions, continued

Factors Affecting Cloning Efficiency

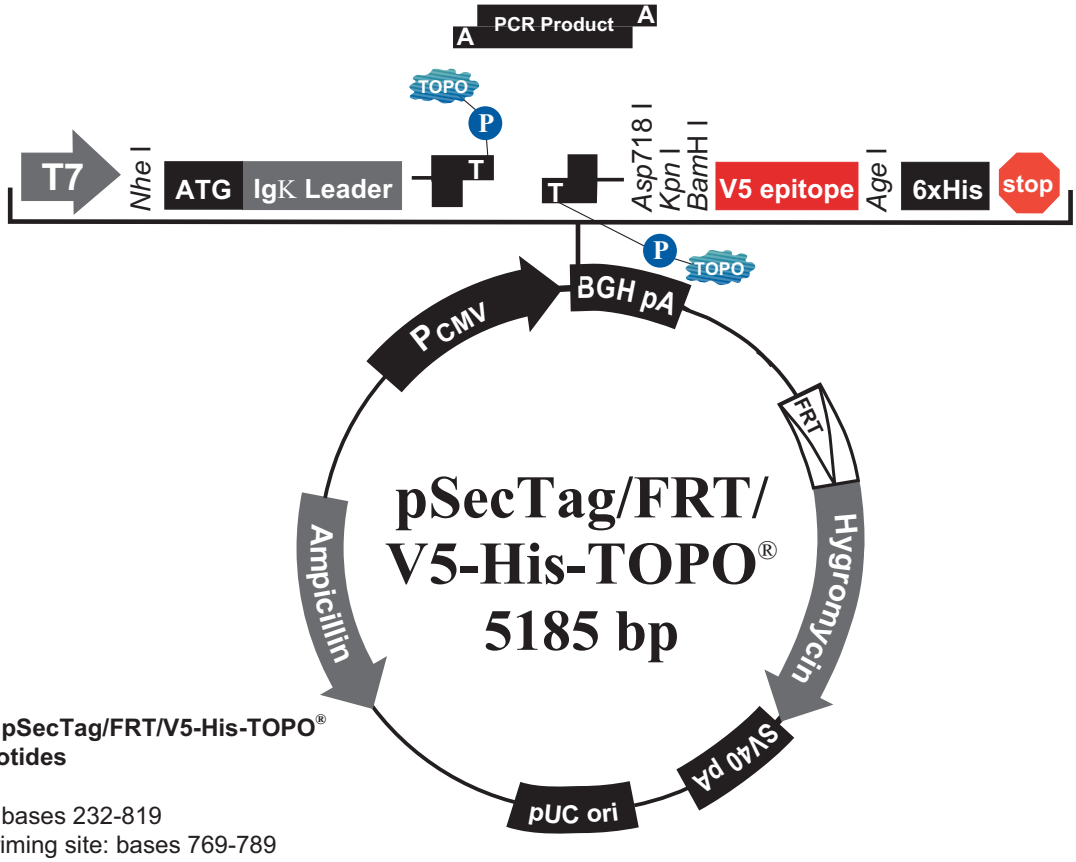
Note that lower cloning efficiencies will result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 85% (or more) cloning efficiency.

Variable	Solution
pH>9 in PCR amplification reaction	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>3 kb)	Increase amount of insert. Or gel-purify as described on page 21.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. Note that you may add up to 4 µl of your PCR to the TOPO® Cloning reaction (page 9).
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 23).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 21) or optimize your PCR. If your template DNA carries an ampicillin marker, carryover into the TOPO® Cloning reaction from the PCR may lead to false positives. Linearize the template DNA prior to PCR to eliminate carryover.
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	*Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).

pSecTag/FRT/V5-His-TOPO® Vector

Map

The figure below summarizes the features of the pSecTag/FRT/V5-His-TOPO® vector (5185 bp). The vector is supplied linearized between nucleotides 1012 and 1013 (TOPO® Cloning site). For a more detailed explanation of each feature, see the next page. **The complete sequence of pSecTag/FRT/V5-His-TOPO® is available from our Web site (www.invitrogen.com) or from Technical Service (see page 30).**



Comments for pSecTag/FRT/V5-His-TOPO®
5185 nucleotides

CMV promoter: bases 232-819
CMV forward priming site: bases 769-789
T7 promoter/priming site: bases 863-882
IgK secretion signal: bases 905-967
TOPO® Cloning site: bases 1012-1013
V5 epitope: bases 1046-1087
Polyhistidine (6xHis) region: bases 1097-1114
BGH reverse priming site: bases 1137-1154
BGH polyadenylation signal: bases 1143-1367
FRT site: bases 1651-1698
Hygromycin resistance gene (no ATG): bases 1706-2725
SV40 early polyadenylation signal: bases 2858-2988
pUC origin: bases 3371-4044 (complementary strand)
bla promoter: bases 5050-5148 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4189-5049 (complementary strand)

continued on next page

pSecTag/FRT/V5-His-TOPO® Vector, continued

Features of pSecTag/FRT/V5-His-TOPO®

pSecTag/FRT/V5-His-TOPO® is a 5185 bp vector that expresses your gene of interest under the control of the human CMV promoter. The table below describes the relevant features of pSecTag/FRT/V5-His-TOPO®. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Allows high-level expression of your gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
CMV Forward priming site	Allows sequencing in the sense orientation
T7 promoter/priming site	Allows *in vitro* transcription in the sense orientation and sequencing through the insert
Murine Ig κ-chain secretion signal	Directs secreted expression of the recombinant fusion protein (Coloma *et al.*, 1992)
TOPO® Cloning site	Allows insertion of your PCR product in frame with the C-terminal peptide containing the V5 epitope and polyhistidine (6xHis) tag
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 Antibody (Catalog no. R960-25) or Anti V5-HRP Antibody (Catalog no. R961-25) (Southern *et al.*, 1991)
Polyhistidine (6xHis) tag	Allows s purification of your recombinant protein on metal-chelating resin such as ProBond™. In addition, the C-terminal 6xHis tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His(C-term)-HRP Antibody (Catalog no. R931-25) (Lindner *et al.*, 1997)
BGH Reverse priming site	Allows sequencing of the non-coding strand
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and poly-adenylation of mRNA (Goodwin and Rottman, 1992)
Flp Recombination Target (FRT) site	Encodes a 34 bp (+14 bp of non-essential) sequence that serves as the binding and cleavage site for Flp recombinase (Gronostajski and Sadowski, 1985; Jayaram, 1985; Senecoff *et al.*, 1985)
Hygromycin resistance gene (no ATG)	Allows selection of stable transfectants in mammalian cells (Gritz and Davies, 1983) when brought in frame with a promoter and an ATG initiation codon through Flp recombinase-mediated recombination via the FRT site
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

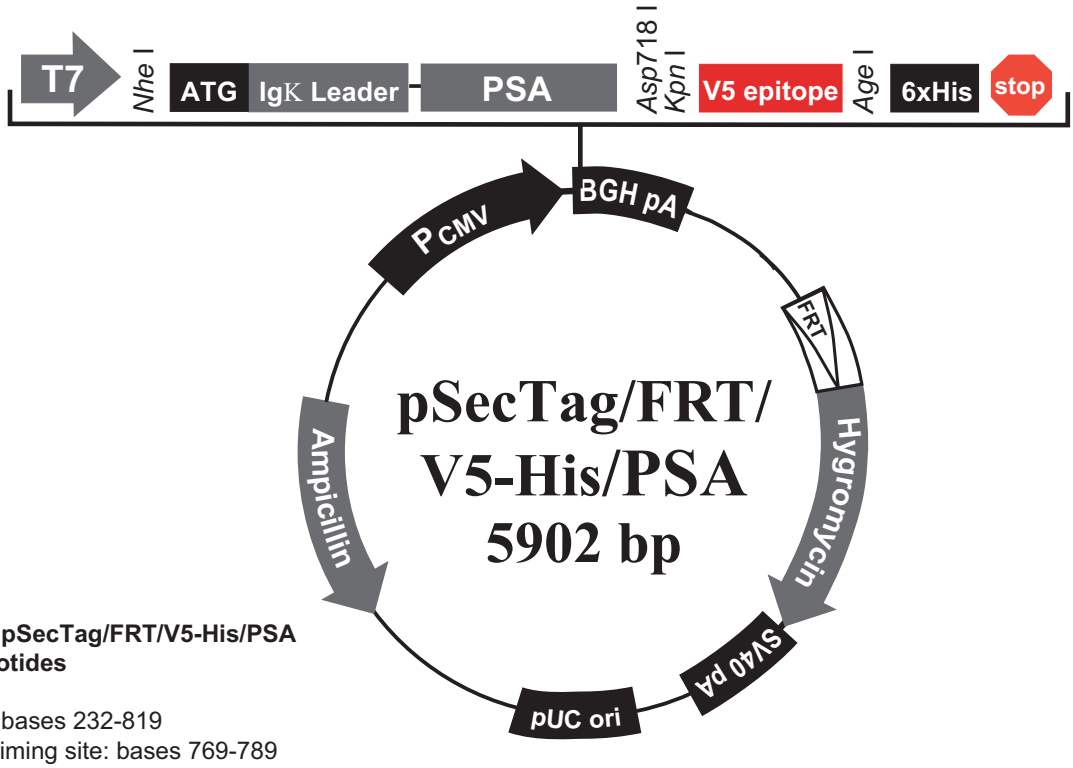
pSecTag/FRT/V5-His/PSA Map

Description

pSecTag/FRT/V5-His/PSA is a 5902 bp control vector expressing prostate specific antigen (PSA). The PSA gene was amplified using PCR and TOPO® Cloned into pSecTag/FRT/V5-His-TOPO®. PSA is expressed as a fusion to the V5 epitope and 6xHis tag. The molecular weight of the fusion protein is approximately 31.5 kDa.

Map

The figure below summarizes the features of the pSecTag/FRT/V5-His/PSA vector. **The complete nucleotide sequence for pSecTag/FRT/V5-His/PSA is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service.** See page 30 for more information.



Comments for pSecTag/FRT/V5-His/PSA 5902 nucleotides

CMV promoter: bases 232-819
CMV forward priming site: bases 769-789
T7 promoter/priming site: bases 863-882
IgK secretion signal: bases 905-967
PSA ORF: bases 1019-1729
V5 epitope: bases 1763-1804
Polyhistidine (6xHis) region: bases 1814-1831
BGH reverse priming site: bases 1854-1871
BGH polyadenylation signal: bases 1860-2084
FRT site: bases 2368-2415
Hygromycin resistance gene (no ATG): bases 2423-3442
SV40 early polyadenylation signal: bases 3575-3705
pUC origin: bases 4088-4761 (complementary strand)
bla promoter: bases 5767-5865 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4906-5766 (complementary strand)

Technical Service

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Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

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Product Specifications

Introduction

This section describes the criteria used to qualify the components in the pSecTag/FRT/V5-His TOPO® TA Expression Kit.

Vectors

The pSecTag/FRT/V5-His supercoiled vector (parental vector of pSecTag/FRT/V5-His-TOPO®) and pSecTag/FRT/V5-His/PSA are qualified by restriction digest with specific restriction enzymes as listed below. Please note that the pSecTag/FRT/V5-His plasmid is qualified by restriction digest prior to adaptation with topoisomerase I, therefore, restriction sites used to qualify the parental vector may no longer be present in the topoisomerase I-adapted vector. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel. The table below lists the restriction enzymes and the expected fragments. The size of the parental pSecTag/FRT/V5-His vector is 5167 bp.

Vector	Restriction Enzyme	Expected Fragments (bp)
pSecTag/FRT/V5-His	*Bam*H I *Eco*R I *Mlu* I *Pvu* II	5167 5167 839, 4328 1804, 3363
pSecTag/FRT/V5-His/PSA	*Bam*H I *Eco*R I *Mlu* I *Pvu* II	741, 5161 5902 1574, 4328 973, 1802, 3125

TOPO® Cloning Efficiency

Once the pSecTag/FRT/V5-His vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on pages 24-25, a 500 bp control PCR product was TOPO® Cloned into pSecTag/FRT/V5-His-TOPO® and subsequently transformed into the One Shot® competent *E. coli* included with the kit. Each lot of vector should yield greater than 85% cloning efficiency.

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 μg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/μg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 μg/ml ampicillin, 25 μg/ml streptomycin, 50 μg/ml kanamycin, or 15 μg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229.

Andrews, B. J., Proteau, G. A., Beatty, L. G., and Sadowski, P. D. (1985). The FLP Recombinase of the 2 Micron Circle DNA of Yeast: Interaction with its Target Sequences. Cell *40*, 795-803.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996). Modulation of Non-Templated Nucleotide Addition by *Taq* DNA Polymerase: Primer Modifications that Facilitate Genotyping. BioTechniques *20*, 1004-1010.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Molec. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Coloma, M. J., Hastings, A., Wims, L. A., and Morrison, S. L. (1992). Novel Vectors for the Expression of Antibody Molecules Using Variable Regions Generated by Polymerase Chain Reaction. J. Imm. Methods *152*, 89-104.

Craig, N. L. (1988). The Mechanism of Conservative Site-Specific Recombination. Ann. Rev. Genet. *22*, 77-105.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Goodwin, E. C., and Rottman, F. M. (1992). The 3´-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J. Biol. Chem. *267*, 16330-16334.

Gritz, L., and Davies, J. (1983). Plasmid-Encoded Hygromycin-B Resistance: The Sequence of Hygromycin-B-Phosphotransferase Gene and its Expression in *E. coli* and *S. Cerevisiae*. Gene *25*, 179-188.

Gronostajski, R. M., and Sadowski, P. D. (1985). Determination of DNA Sequences Essential for FLP-mediated Recombination by a Novel Method. J. Biol. Chem. *260*, 12320-12327.

Jayaram, M. (1985). Two-micrometer Circle Site-specific Recombination: The Minimal Substrate and the Possible Role of Flanking Sequences. Proc. Natl. Acad. Sci. USA *82*, 5875-5879.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129.

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References, continued

Palmer, T. D., Hock, R. A., Osborne, W. R. A., and Miller, A. D. (1987). Efficient Retrovirus-Mediated Transfer and Expression of a Human Adenosine Deaminase Gene in Diploid Skin Fibroblasts from an Adenosine-Deficient Human. Proc. Natl. Acad. Sci. U.S.A. *84*, 1055-1059.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Sauer, B. (1994). Site-Specific Recombination: Developments and Applications. Curr. Opin. Biotechnol. *5*, 521-527.

Senecoff, J. F., Bruckner, R. C., and Cox, M. M. (1985). The FLP Recombinase of the Yeast 2-micron Plasmid: Characterization of its Recombination Site. Proc. Natl. Acad. Sci. USA *82*, 7270-7274.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Shuman, S. (1994). Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. J. Biol. Chem. *269*, 32678-32684.

Shuman, S. (1991). Recombination Mediated by Vaccinia Virus DNA Topoisomerase I in *Escherichia coli* is Sequence Specific. Proc. Natl. Acad. Sci. USA *88*, 10104-10108.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

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